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Activating Mutations in *PIK3CA* Lead to Widespread Modulation of the Tyrosine Phosphoproteome

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Abstract

The human oncogene *PIK3CA* is frequently mutated in human cancers. Two hotspot mutations in *PIK3CA*, E545K and H1047R, have been shown to regulate widespread signaling events downstream of AKT, leading to increased cell proliferation, growth, survival, and motility. We used quantitative mass spectrometry to profile the global phosphotyrosine proteome of isogenic knock-in cell lines containing these activating mutations, where we identified 824 unique phosphopeptides. Although it is well understood that these mutations result in hyperactivation of the serine/threonine kinase AKT, we found a surprisingly widespread modulation of tyrosine phosphorylation levels of proteins in the mutant cells. In the tyrosine kinome alone, 29 tyrosine kinases were altered in their phosphorylation status. Many of the regulated phosphosites that we identified were located in the kinase domain or the canonical activation sites, indicating that these kinases and their downstream signaling pathways were activated. Our study demonstrates that

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Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jproteome.5b00302.

Supporting Information Table 1: A list of phosphoPSM identified. (XLSX)

Supporting Information Table 2: A complete list of phosphorylated peptides identified. (XLSX)

Supporting Information Table 3: A list of the proteins identified in the global proteome analysis. (XLSX)

Supporting Information Table 4: A list of hyperphosphorylated peptides in both Ex9-KI and Ex20-KI cells. (XLSX)

there is frequent and unexpected cross-talk that occurs between tyrosine signaling pathways and serine/threonine signaling pathways activated by the canonical PI3K-AKT axis.

INTRODUCTION

The phosphoinositide 3-kinases (PI3Ks) are a family of lipid kinases that regulate a number of important biological processes including cell growth, survival, proliferation, and differentiation.¹ Class I PI3Ks are heterodimers composed of a regulatory subunit (p85) and a catalytic subunit (p110) that transduce signals from receptors such as G-protein-coupled receptors and receptor tyrosine kinases. Upon growth factor stimulation, the SH2 domains of the p85 subunit bind to the phosphorylated tyrosine residue of the receptors, recruiting PI3K to the membrane. This binding results in the release of inhibition of p85 on the lipid kinase activity of p110, which is then free to phosphorylate the phosphatidylinositol (4,5)-bispophosphate (PIP₂) phospholipid to generate phosphatidylinositol (3,4,5)-trisphosphate (PIP₃).² The accumulation of PIP₃ results in membrane recruitment and activation of PH-domain containing proteins such as PDK1 and AKT. The activation of AKT initiates a slew of signaling events that ultimately result in cell proliferation, survival, growth, and motility. The mitogenic effects from the activation of this pathway is the reason why *PIK3CA*, the gene that encodes p110 α , has been found to be frequently mutated in human cancers.³ Many of the mutations in this gene result in the gain of function of PI3K to confer cells with an oncogenic advantage.

A majority of phosphorylation events in cells occur on serine and threonine residues of proteins with a very small fraction occurring on tyrosine residues. Even though tyrosine phosphorylation accounts for a minority of total phosphorylation, tyrosine kinases play a disproportionately large role in diseases, especially in cancer. More than half of the 90 tyrosine kinases identified in the human proteome have been implicated in cancer through gain-of-function mutations, gene amplification, or overexpression and have become attractive therapeutic targets. We have previously profiled the serine/threonine phosphoproteome of cells containing two hotspot mutations in *PIK3CA*, E545K and H1047R.⁴ In the current study, we sought to profile the tyrosine phosphoproteome changes that result from these activating mutations in *PIK3CA*. We performed phosphotyrosine profiling of these isogenic cell lines, where we identified 824 phosphopeptides derived from 343 proteins. We observed modulation of important biological processes that include cytoskeletal migration pathways and kinase regulated signaling. 127 of the identified

phosphopeptides belong to 34 tyrosine kinases, with 29 of these showing upregulation or downregulation of phosphorylation levels in the mutant knock-in cell lines. This widespread modulation of tyrosine kinome indicates that there is a high degree of crosstalk between tyrosine kinase and serine/threonine kinase signaling pathways resulting from activation of the PI3K-AKT pathway.

METHODS

Reagents

Antiphosphotyrosine mouse mAb (pTyr-1000) beads were purchased from Cell Signaling Technology (Danvers, MA). TPCK-treated trypsin was obtained from Worthington Biochemical (Lakewood, NJ). DMEM/F12 with and without lysine and arginine, fetal bovine serum (FBS), L-glutamine, and antibiotics were purchased from Invitrogen (Carlsbad, CA). SILAC amino acids, $^{13}\text{C}_6$ -lysine, $^{13}\text{C}_6$ -arginine, $^2\text{H}_4$ -lysine, $^{13}\text{C}_6$ -arginine, $^{13}\text{C}_6$ $^{15}\text{N}_2$ -lysine, $^{13}\text{C}_6$ $^{15}\text{N}_4$ -arginine were purchased from Cambridge Isotope Laboratories (Andover, MA). All other reagents used in this study were from Fisher Scientific (Pittsburgh, PA).

Cell Culture

PIK3CA mutant knock-in breast epithelial cell lines MCF10A (hereafter referred to as Ex9-KI and Ex20-KI) were established as previously described.⁵ For experiments here, Ex9-KI cells, Ex20-KI cells, and MCF10A parental breast epithelial cells were cultured following a protocol similar to that previously described.⁴ All three cell lines (Ex9-KI, Ex20-KI, and MCF10A) were grown in 5% CO_2 at 37 °C. Cell culture media consisted of DMEM/F12 (1:1) supplemented with 5% horse serum, 10 $\mu\text{g}/\text{mL}$ insulin (Roche), 0.5 $\mu\text{g}/\text{mL}$ hydrocortisone (Sigma), and 100 ng/mL cholera toxin (Sigma) and either 20 ng/mL EGF (for MCF10A parental cells) or 0.2 ng/mL EGF (for Ex9-KI and Ex20-KI cells).

Cell Line Labeling

Three-state stable isotopic labeling by amino acids in cell culture (SILAC) of MCF10A parental cells and Ex9-KI and Ex20-KI cells was performed. In brief, cells were cultured in DMEM/F12 (1:1) SILAC media deficient in both L-lysine and L-arginine (Thermo Fisher Scientific). Ex20-KI cell culture media was supplemented with light lysine (K) and light arginine (R) to facilitate incorporation of the “light” labels. Ex9-KI cell culture media was supplemented with $^2\text{H}_4$ -K and $^{13}\text{C}_6$ -R to facilitate incorporation of the “medium” labels. Parental MCF10A cell culture media was supplemented with $^{13}\text{C}_6$ $^{15}\text{N}_2$ -K and $^{13}\text{C}_6$ $^{15}\text{N}_4$ -R to facilitate “heavy”-state labeling. Prior to harvest, all cell lines were trypsinized, washed, and seeded at 80% confluency in DMEM/F12 basal media containing only 5% horse serum and grown overnight. Two sets of three-state SILAC-labeled cells were prepared as replicates for downstream mass spectrometry analysis.

In-Solution Trypsin Digestion

Following cell culture, peptides were prepared in an in-solution tryptic digestion protocol with slight modifications.⁶ In brief, cells were lysed in urea lysis buffer (20 mM HEPES pH 8.0, 9 M urea, 1 mM sodium orthovanadate, 2.5 mM sodium pyrophosphate, 1 mM β -glycerophosphate, and 5 mM sodium fluoride), sonicated, and then cleared by centrifugation

at 15 000g at 4 °C for 20 min. As determined by BCA assay, 20 mg protein from each SILAC-labeled cell lysate was mixed. The resultant mixture was then reduced with 5 mM dithiothreitol and alkylated with 10 mM iodoacetamide. For in-solution tryptic digestion, the resulting protein extracts were diluted in 20 mM HEPES pH 8.0 to a final concentration lower than 2 M urea incubated with 1 mg/mL TPCK-treated trypsin on an orbital shaker at 25 °C overnight. Protein digests were acidified with 1% trifluoroacetic acid (TFA) to quench the digestion reaction and then subjected to centrifugation at 2000g at room temperature for 5 min. The resulting supernatants were desalted using SepPak C₁₈ cartridge. Eluted peptides were lyophilized to dryness prior to phosphotyrosine peptide enrichment.

Basic Reversed-Phase Liquid Chromatography (RPLC)

For the total proteome analysis, basic RPLC was carried out as previously described.⁷ Agilent 1100 offline LC system was used for bRPLC fractionation, which includes a binary pump, VWD detector, and an automatic fraction collector. In brief, lyophilized samples were reconstituted in solvent A (10 mM triethylammonium bicarbonate, pH 8.5) and loaded onto XBridge C₁₈, 5 μ m 250 $\times$ 4.6 mm column (Waters, Milford, MA). Peptides were resolved using a gradient of 3 to 50% solvent B (10 mM triethylammonium bicarbonate in acetonitrile, pH 8.5) over 50 min and then kept at 90% for another 10 min. A total of 96 fractions were collected, and these were concatenated into 12 fractions. Samples were then dried in vacuum and stored in –80 °C freezer prior to LC–MS/MS analysis.

Immunoaffinity Purification of Phosphotyrosine Peptides

Immunoaffinity purification (IAP) of phosphotyrosine peptides was performed as previously described.⁶ In brief, following lyophilization, desalted lyophilized tryptic peptides were reconstituted in 1.4 mL of IAP buffer (50 mM MOPS pH 7.2, 10 mM sodium phosphate, 50 mM NaCl) and subjected to centrifugation at 2000g at room temperature for 5 min. Prior to IAP, antiphosphotyrosine antibody beads (pY1000, Cell Signaling Technology) were washed with IAP buffer once. The reconstituted peptide mixtures were then incubated with antiphosphotyrosine antibody beads on a rotator at 4 °C for 30 min. Samples were then centrifuged at 1500g for 1 min and supernatant was removed. The beads were washed twice with IAP buffer and then twice with water. Residual water was removed. Phosphopeptides were eluted from the antibody beads by acidifying the bead mixture at room temperature with 0.1% TFA. Phosphopeptides eluents were desalted with C18 STAGE tips, vacuum-dried, and stored at –80 °C prior to LC–MS/MS analysis.

Liquid Chromatography Tandem Mass Spectrometry

Data-dependent LC–MS/MS analysis of phosphopeptides enriched by IAP was performed with an LTQ-Orbitrap Elite mass spectrometer (Thermo Fisher Scientific) coupled to a nanoliquid chromatography system (Proxeon, Easy Nano-LC). During each LC–MS/MS run, 10 μ L of reconstituted peptide solution was injected onto a nano-c18 reversed-phase column (10 cm $\times$ 75 μ m, Magic C18 AQ 5 μ m, 120 Å). Peptides were then fractionated across a 90 min linear reversed-phase HPLC gradient (from 5 to 60% acetonitrile). High-resolution precursor scans (FTMS) were acquired within the Orbitrap analyzer across a mass range of 350–1700 Da (with 120 000 resolution at 400 *m/z*). The 15 most abundant

precursor ions from each precursor scan were selected for high collision dissociation (HCD) fragmentation (isolation width of 1.90 m/z ; 32% normalized collision energy; and activation time of 0.1 ms). High-resolution MS/MS spectra were acquired (at 30 000 resolution at 400 m/z) on the Orbitrap analyzer following fragmentation.

Mass Spectrometry Data Analysis

The Proteome Discoverer (v 1.4; Thermo Fisher Scientific) software package was used to facilitate downstream protein identification and quantitation. All acquired mass spectrometric data were searched within the Proteome Discoverer interface using both Mascot (version 2.2.0) and SEQUEST search algorithms against Human RefSeq database v 50 (containing 35,478 entries). For both algorithms, search parameters were as follows: a maximum of two missed cleavages; a fixed modification of carbamidomethylation; variable modifications of N-terminal acetylation, oxidation at methionine, phosphorylation at serine, threonine, and tyrosine and SILAC labeling $^{13}\text{C}_6$ $^{15}\text{N}_2$ -lysine, $^2\text{H}_4$ -lysine, $^{13}\text{C}_6$ -arginine and $^{13}\text{C}_6$, $^{15}\text{N}_2$ -arginine; MS tolerance of ± 10 ppm; and MS/MS tolerance of ± 0.1 Da. The Mascot and SEQUEST score cut-offs were set to a false discovery rate of 1% at the peptide level. The q values for the peptides were calculated using the Percolator algorithm within the Proteome Discover suite. The peptide quantification was performed using the algorithms available within the precursor ion quantifier node. Quantitation was determined based on area under the curve measurements from the extracted ion chromatograms for each precursor ion. The probability that an identified phosphorylation was modifying each specific Ser/Thr/Tyr residue on each identified phosphopeptide was determined from the PhosphoRS algorithm.⁸ We averaged and normalized the intensities of the phosphopeptides identified in the two replicate experiments that were carried out. Total sum intensities of all phosphopeptides for each SILAC label were used to normalize the phosphopeptide abundance. 1.5-fold cutoff was selected for hyperphosphorylation, and a 0.67-fold cutoff was selected to denote hypophosphorylation. All mass spectrometry proteomics data associated with this project have been deposited to the ProteomeXchange Consortium (<http://proteomecentral.proteomexchange.org>) via the PRIDE partner repository with the data set identifier PXD001460.

Western Blot Analysis

All cell lines used for Western blot analyses were cultured in regular medium with light amino acids. Prior to harvest, cells were seeded overnight in medium containing only 5% horse serum. Cells were harvested and lysed in modified RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 0.25% sodium deoxycholate, and 1 mM sodium orthovanadate in the presence of protease inhibitors). Whole cell protein extracts were denatured and separated in NuPAGE gels (Invitrogen), transferred to nitrocellulose membranes, and probed with primary and horseradish-peroxidase-conjugated secondary antibodies. The primary antibodies used were antiphospho-EGFR Y1173 (4407; Cell Signaling Technology), anti-EGFR (2232; Cell Signaling Technology), antiphospho-EPHA2 Y588 (12677; Cell Signaling Technology), anti-EPHA2 (6997; Cell Signaling Technology), antiphospho-MET Y1003 (3135; Cell Signaling Technology), anti-MET (3148; Cell Signaling Technology), antiphospho-EFNB1 Y324 (OAAF00520; Aviva Systems Biology), anti-EFNB1 (ARP46450_P050; Aviva Systems Biology), phospho-HER2 Y877 (2265-1;

Epitomics), anti-HER2 (2165; Cell Signaling Technology), and anti- β -ACTIN (A5316, Sigma).

RESULTS AND DISCUSSION

Phosphotyrosine Profiling of Mutant *PIK3CA* Knock-in Cells Reveals Widespread Modulation of the Tyrosine Phosphoproteome

The p110 α subunit of PI3K is composed of an N-terminal p85 binding domain, a Ras binding domain, a C2 domain, a helical domain, and a kinase domain (Figure 1A).⁹ The gene encoding p110 α , *PIK3CA*, has been shown to be frequently mutated in human cancers.³ The cBioPortal for Cancer Genomics online tool (www.cbioportal.org), which compiles sequencing data from large-scale sequencing studies of human cancers, revealed three hotspot mutations in this gene (Figure 1A).^{10,11} Two of these mutations occur in exon 9 of the gene (E542K and E545K), which codes for the helical domain, and another mutation occurs in exon 20 of the gene (H1047R), which codes for the kinase domain. These single amino acid mutations result in a gain of function of PI3K, which ultimately leads to activation of AKT signaling and inducing growth factor and anchorage-independent growth, cell motility and tumor formation *in vivo*.^{12–14} We previously established cell lines that contain the E545K and H1047R mutations (hereafter referred to as Ex9-KI and Ex20-KI, respectively) using gene targeting to knock-in these mutations into the spontaneously immortalized, nontumorigenic breast epithelial cell line MCF10A.⁵ A breast epithelial cell line model is especially relevant because *PIK3CA* has been shown to be the most frequently mutated gene across all subtypes of breast tumors.^{15,16} In a previous study, we have carried out phosphoserine/threonine profiling of these isogenic cell lines using TiO₂ phosphopeptide enrichment.⁴ We found multiple receptor and nonreceptor tyrosine kinases including EGFR, EPHA2, and PTK2 to be hyperphosphorylated in the mutant cells, which led us to hypothesize that tyrosine kinases, and subsequently their downstream signaling pathways, are activated in *PIK3CA* mutant cells. In the current study, we aimed to perform an in-depth analysis of tyrosine kinase signaling pathways by specifically enriching for tyrosine phosphorylated peptides. To this end, we performed SILAC (stable isotope labeling by amino acids in cell culture) labeling on the mutant knock-in cell lines along with the parental MCF10A to allow for accurate quantitation of phosphorylation levels. The three state-SILAC experiment was carried out by mixing equal amounts of protein lysates from Ex20-KI cells labeled with light SILAC medium, Ex9-KI (medium), and parental MCF10A (heavy). The mixture was digested with trypsin, and the tryptic peptides were then desalted using the C₁₈ cartridge. Phosphotyrosine enrichment of the phosphopeptides was carried out using the antiphosphotyrosine antibody pulldown prior to LC-MS/MS analysis (Figure 1B).

From this profiling, we identified 824 phosphopeptides from 343 proteins (Supporting Information Tables 1 and 2). A majority of these phosphopeptides (651) are phosphorylated on tyrosine residues and a smaller fraction on serine/threonine residues (173). Most of these phosphopeptides showed similar phosphorylation pattern in both Exon 9 and Exon 20 (Figure 2A). We observed a global elevation of protein phosphorylation level in the mutant knock-in compared with the parental cells, where more peptides showed upregulation in phosphorylation levels compared with downregulation (Figure 2B). This indicates that

PIK3CA mutations lead to a global increase in protein phosphorylation levels and hence profoundly impact the tyrosine signaling pathways. To investigate the stoichiometry of the observed phosphorylation-site changes, we performed a global proteome analysis of the same cell lines. We observed that most of the proteome did not change in the knock-in cell lines, indicating that tyrosine phosphoproteome modulation is mostly due to the activation of tyrosine kinases (Figure 2D, Supporting Information Table 3). In our previous work,⁴ we identified 166 phosphotyrosine-containing peptides out of 8075 phosphopeptides identified. In the current study, we identified 651 phosphotyrosine-containing peptides out of 824 phosphopeptides identified, a number four times larger than our previous study. More importantly, none of the phosphotyrosine-containing peptides identified in our previous study belong to any of the tyrosine kinases in the human proteome (11 tyrosine kinases were identified from phosphoserine/threonine-containing peptides), whereas our current study identified 34 tyrosine kinases, with 29 of these showing alterations in their phosphorylation status. This indicates the necessity of phosphotyrosine peptide-specific enrichment to study the tyrosine phosphoproteome modulation in the cells.

The peptides that were found to be hyperphosphorylated in both mutant cell types are listed in Supporting Information Table 4. Analysis using KEGG pathway database showed that many of these hyperphosphorylated proteins are involved in important biological processes such as cell motility, which includes pathways in regulation of actin cytoskeleton, focal adhesion, tight and adherens junctions, as well as kinase regulated pathways such as ErbB, insulin, and VEGF signaling pathways (Figure 2C). Of particular note, many of these pathways were also found to be regulated through our phosphoserine/threonine profiling in our previous study, supporting the robustness of our mass spectrometry analysis.⁴ Activation of these pathways in the *PIK3CA* mutant cells could potentially explain why these cells are able to proliferate in the absence of growth factors and are significantly more invasive than the wild-type cells.

Activating *PIK3CA* Mutations Modulate the Tyrosine Kinome

Of the 343 proteins that we identified from our profiling, 63 are protein kinases. 34 of these belong to the tyrosine kinase family where 16 are receptor tyrosine kinases and 18 are nonreceptor tyrosine kinases. We mapped the identified kinases onto the phylogenetic tree of the human kinome (Figure 3). A majority of the phosphopeptides from these kinases showed either an increase or decrease in phosphorylation levels, suggesting that the activity of these kinases is regulated. This regulation signifies that the signaling pathways downstream of these kinases, for example, the focal adhesion pathway and ErbB signaling pathway (Figure 2C), are also modulated. More kinases were found to have increased levels of phosphorylation compared with decreased levels of phosphorylation. Figure 4 shows representative spectra of phosphopeptides belonging to these kinases. As shown in this Figure, Ex9-KI and Ex20-KI cells have increased phosphorylation compared with the parental MCF10A cells. Many of the identified sites on these hyperphosphorylated kinases have been implicated in oncogenic transformation or metastasis. For instance, phosphorylation of EGFR at Y1110 has been reported to stimulate cancer cell invasion, motility, and migration.¹⁷ Phosphorylation of Y588 of EPHA2 has been reported to be critical for vascular assembly and tumor angiogenesis.¹⁸ Thus, the phosphorylation of these

kinases could contribute to the oncogenic effects that are observed in cells with *PIK3CA* mutation.

One possible mechanism of how these kinases were activated is through direct phosphorylation by AKT. Both EGFR and EPHA2 have been demonstrated to be substrates of AKT. EGFR S229 phosphorylation by AKT was shown to contribute to drug resistance, and EPHA2 S897 phosphorylation by AKT was found to stimulate cell migration.^{19,20} AKT phosphorylation on these sites could lead to phosphorylation of other tyrosine residues on EGFR and EPHA2 through autophosphorylation and dimerization. The activated EGFR and EPHA2 could heterodimerize with other family members such as ERBB2,^{21,22} leading to the hyperphosphorylation of many members of epidermal growth factor receptor family and ephrin receptor family, which was observed in the *PIK3CA* mutant cells. Another possible mechanism of tyrosine kinase activation in the mutant cell is through direct activation by PIP₃. There are four tyrosine kinases that contain the pleckstrin homology domain that can bind to PIP₃, namely, BMX, BTK, TEC, and TIK. Studies have demonstrated that mutant *PIK3CA* could activate BMX, which could, in turn, directly phosphorylate STAT3 on Y705.^{23,24} In our profiling, we found STAT3 Y705 to be hyperphosphorylated 2.3-fold in Ex9-KI and 1.6-fold in Ex20-KI cells, suggesting that this mechanism is also active in the mutant cells; however, the hyperphosphorylation of many of the tyrosine kinases we identified could not be explained simply by activation through PIP₃ or AKT pathway. Thus, this indicates that there are activations of various tyrosine kinase signaling through cross-talks and other mechanisms that are still not well-elucidated.

Site-Specific Analysis of Tyrosine Kinase Phosphorylation Regulated by *PIK3CA* Mutations

To understand the effects of regulation of phosphorylation of the tyrosine kinases, we mapped the phosphorylation sites we identified in our profiling onto the protein structure. The receptor tyrosine kinases that exhibit regulation of phosphorylation levels are shown in Figure 5A, and the nonreceptor tyrosine kinases are depicted in Figure 5B. Receptor tyrosine kinases are composed of a large extracellular domain that binds to ligands, a transmembrane domain, and a cytoplasmic tail, which contains the tyrosine kinase domain. All of the phosphorylation sites we identified were localized on the cytoplasmic tail, indicating that regulation of activity occurs intracellularly. Nonreceptor tyrosine kinases typically contain a tyrosine kinase domain along with other domains such as SH2 and SH3, which allow interaction and binding to other proteins. In both receptor and nonreceptor tyrosine kinases, we observed that many of the regulated phosphorylation sites lie within the tyrosine kinase domain. The phosphorylation of many of the sites in this domain has been shown to be important for activity of the corresponding kinases. This includes Y877 of ERBB2, better known as HER2,^{25–27} Y869 of EGFR,^{28,29} Y772 of EPHA2,¹⁸ Y1189 and Y1190 of INSR,³⁰ Y714 of FER,³¹ and Y347 of TNK2,^{32,33} all of which were found to be hyperphosphorylated in the mutant cells. We also observed many known regulatory sites outside of the tyrosine kinase domain to be hyperphosphorylated in the mutant cells. These include the C-terminal Y1197 autophosphorylation site of EGFR, which has been reported to be important for its enzymatic activity in addition to serving as a docking site for substrates such as PLC- γ and Shc.^{34–36} Phosphorylation of the juxtamembrane region of

EPHB2 has been demonstrated to stimulate its catalytic activity by removing the inhibitory conformation of this region and serving as recruitment sites for proteins containing SH2 domains such as Ras-GAP.^{37,38} Similarly, phosphorylation of Y323 of SYK has been reported to be required for the interaction with its substrate Cbl and the maximal tyrosine phosphorylation of Cbl.³⁹ Taken together, the preponderance of regulated sites identified in the kinase domain and regulatory regions signifies that these kinases and their downstream signaling pathways are activated. In addition to these well-studied sites, there are other sites that were found to be regulated in the mutant cells, whose functions are still unknown. Determining the significance of these phosphorylation sites in inducing oncogenic effects downstream of PI3K will require additional studies.

Validation of the Phosphoproteomic Screen by Western Blot Analysis

To validate our phosphoproteomic screen, we performed Western blot analysis using antibodies against phosphorylated sites of proteins identified in our global profiling, namely, EGFR, EPHA2, MET, EFNB1, and ERBB2. For each of these antibodies, we showed through Western blot that the levels of phosphorylation are consistent with our mass spectrometry results (Figure 6). For example, we found EGFR Y1197 and EPHA2 Y588 to be hyperphosphorylated at about 2-fold in Ex9-KI and Ex20-KI cells, and we observed a consistent increase in signal in our Western blot. ERBB2 Y877 on the contrary showed elevation in only Ex20-KI (2-fold) but not in Ex9-KI (1.1-fold), and we confirmed these data with our Western blot. MET Y1021 did not show marked elevation of phosphorylation in our profiling data in Ex9-KI (1.3-fold) and Ex20-KI (1.1-fold), which was supported by the Western blot analysis. We also performed Western blot of total protein for each of these proteins, and we observed similar levels of protein expression in each of MCF10A parental, Ex9-KI, and Ex20-KI cells (except for HER2, where we observed a slight decrease in protein levels in mutant cells even though the phosphorylation levels increased). This indicates that these proteins are hyperphosphorylated through activation of kinases and not as a result of transcriptional/translational regulation. Thus, we have demonstrated through an orthogonal method that our mass spectrometry profiling data are accurate.

CONCLUSIONS

Through phosphoproteomic profiling of cells with a single amino acid change in the *PIK3CA* gene, we demonstrate that there is a widespread modulation of the tyrosine phosphoproteome due to these activating mutations. Even though these mutations have been shown to result primarily in the hyperactivation of pathways downstream of the serine/threonine kinase AKT, our results clearly indicate that tyrosine signaling pathways are also widely affected. The activation of a few tyrosine kinases in the mutant *PIK3CA* cells could be the result of direct binding to PIP₃ or direct phosphorylation by AKT; however, many others could not be explained through these mechanisms as phosphotyrosine signaling is generally not studied in the context of serine/threonine kinases. This suggests that there are hitherto unknown mechanisms of crosstalk that occur between these pathways. Our profiling study should serve as a potentially useful resource for research as well as clinical studies involving development of novel therapeutic targets.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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REFERENCES

1. Vivanco I, Sawyers CL. The phosphatidylinositol 3-Kinase–AKT pathway in human cancer. *Nat. Rev. Cancer.* 2002; 2(7):489–501. [PubMed: 12094235]
2. Yu J, Zhang Y, McIlroy J, Rordorf-Nikolic T, Orr GA, Backer JM. Regulation of the p85/p110 Phosphatidylinositol 3'-Kinase: Stabilization and Inhibition of the p110 α Catalytic Subunit by the p85 Regulatory Subunit. *Mol. Cell. Biol.* 1998; 18(3):1379–1387. [PubMed: 9488453]
3. Samuels Y, Wang Z, Bardelli A, Silliman N, Ptak J, Szabo S, Yan H, Gazdar A, Powell SM, Riggins GJ, et al. High Frequency of Mutations of the PIK3CA Gene in Human Cancers. *Science.* 2004; 304(5670):554–554. [PubMed: 15016963]
4. Wu X, Renuse S, Sahasrabudhe NA, Zahari MS, Chaerkady R, Kim M-S, Nirujogi RS, Mohseni M, Kumar P, Raju R. Activation of diverse signalling pathways by oncogenic PIK3CA mutations. *Nat. Commun.* 2014; 5:4961. [PubMed: 25247763]
5. Gustin JP, Karakas B, Weiss MB, Abukhdeir AM, Lauring J, Garay JP, Cosgrove D, Tamaki A, Konishi H, Konishi Y, et al. Knockin of mutant PIK3CA activates multiple oncogenic pathways. *Proc. Natl. Acad. Sci. U. S. A.* 2009; 106(8):2835–2840. [PubMed: 19196980]
6. Rush J, Moritz A, Lee KA, Guo A, Goss VL, Spek EJ, Zhang H, Zha X-M, Polakiewicz RD, Comb MJ. Immunoaffinity profiling of tyrosine phosphorylation in cancer cells. *Nat. Biotechnol.* 2005; 23(1):94–101. [PubMed: 15592455]
7. Wang Y, Yang F, Gritsenko MA, Wang Y, Clauss T, Liu T, Shen Y, Monroe ME, Lopez-Ferrer D, Reno T, et al. Reversed-phase chromatography with multiple fraction concatenation strategy for proteome profiling of human MCF10A cells. *Proteomics.* 2011; 11(10):2019–2026. [PubMed: 21500348]
8. Taus T, Köcher T, Pichler P, Paschke C, Schmidt A, Henrich C, Mechtler K. Universal and Confident Phosphorylation Site Localization Using phosphoRS. *J. Proteome Res.* 2011; 10(12): 5354–5362. [PubMed: 22073976]
9. Amzel LM, Huang C-H, Mandelker D, Lengauer C, Gabelli SB, Vogelstein B. Structural comparisons of class I phosphoinositide 3-kinases. *Nat. Rev. Cancer.* 2008; 8(9):665–669. [PubMed: 18633356]
10. Gao J, Aksoy BA, Dogrusoz U, Dresdner G, Gross B, Sumer SO, Sun Y, Jacobsen A, Sinha R, Larsson E, et al. Integrative Analysis of Complex Cancer Genomics and Clinical Profiles Using the cBioPortal. *Sci. Signaling.* 2013; 6(269):p11–p11.
11. Cerami E, Gao J, Dogrusoz U, Gross BE, Sumer SO, Aksoy BA, Jacobsen A, Byrne CJ, Heuer ML, Larsson E, et al. The cBio Cancer Genomics Portal: An Open Platform for Exploring Multidimensional Cancer Genomics Data. *Cancer Discovery.* 2012; 2(5):401–404. [PubMed: 22588877]
12. Zhao JJ, Liu Z, Wang L, Shin E, Loda MF, Roberts TM. The oncogenic properties of mutant p110 α and p110 β phosphatidylinositol 3-kinases in human mammary epithelial cells. *Proc. Natl. Acad. Sci. U. S. A.* 2005; 102(51):18443–18448. [PubMed: 16339315]

13. Kang S, Bader AG, Vogt PK. Phosphatidylinositol 3-kinase mutations identified in human cancer are oncogenic. *Proc. Natl. Acad. Sci. U. S. A.* 2005; 102(3):802–807. [PubMed: 15647370]
14. Ikenoue T, Kanai F, Hikiba Y, Obata T, Tanaka Y, Imamura J, Ohta M, Jazag A, Guleng B, Tateishi K, et al. Functional Analysis of PIK3CA Gene Mutations in Human Colorectal Cancer. *Cancer Res.* 2005; 65(11):4562–4567. [PubMed: 15930273]
15. The Cancer Genome Atlas Network. Comprehensive molecular portraits of human breast tumours. *Nature.* 2012; 490(7418):61–70. [PubMed: 23000897]
16. Bachman KE, Argani P, Samuels Y, Silliman N, Ptak J, Szabo S, Konishi H, Karakas B, Blair BG, Lin C, et al. The PIK3CA gene is mutated with high frequency in human breast cancers. *Cancer Biol. Ther.* 2004; 3(8):772–775. [PubMed: 15254419]
17. Cardoso AP, Pinto ML, Pinto AT, Oliveira MI, Pinto MT, Gonçalves R, Relvas JB, Figueiredo C, Seruca R, Mantovani A, et al. Macrophages stimulate gastric and colorectal cancer invasion through EGFR Y1086, c-Src, Erk1/2 and Akt phosphorylation and smallGTPase activity. *Oncogene.* 2014; 33(16):2123–2133. [PubMed: 23644655]
18. Fang WB, Brantley-Sieders DM, Hwang Y, Ham A-JL, Chen J. Identification and Functional Analysis of Phosphorylated Tyrosine Residues within EphA2 Receptor Tyrosine Kinase. *J. Biol. Chem.* 2008; 283(23):16017–16026. [PubMed: 18387945]
19. Miao H, Li D-Q, Mukherjee A, Guo H, Petty A, Cutter J, Basilion JP, Sedor J, Wu J, Danielpour D, et al. EphA2 Mediates Ligand-Dependent Inhibition and Ligand-Independent Promotion of Cell Migration and Invasion via a Reciprocal Regulatory Loop with Akt. *Cancer Cell.* 2009; 16(1):9–20. [PubMed: 19573808]
20. Huang W-C, Chen Y-J, Li L-Y, Wei Y-L, Hsu S-C, Tsai S-L, Chiu P-C, Huang W-P, Wang Y-N, Chen C-H, et al. Nuclear Translocation of Epidermal Growth Factor Receptor by Akt-dependent Phosphorylation Enhances Breast Cancer-resistant Protein Expression in Gefitinib-resistant Cells. *J. Biol. Chem.* 2011; 286(23):20558–20568. [PubMed: 21487020]
21. Wada T, Qian X, Greene MI. Intermolecular association of the p185neu protein and EGF receptor modulates EGF receptor function. *Cell.* 1990; 61(7):1339–1347. [PubMed: 1973074]
22. Goldman R, Ben Levy R, Peles E, Yarden Y. Heterodimerization of the erbB-1 and erbB-2 receptors in human breast carcinoma cells: a mechanism for receptor transregulation. *Biochemistry.* 1990; 29(50):11024–11028. [PubMed: 1980216]
23. Guryanova OA, Wu Q, Cheng L, Lathia JD, Huang Z, Yang J, MacSwords J, Eyler CE, McLendon RE, Heddleston JM, et al. Nonreceptor Tyrosine Kinase BMX Maintains Self-Renewal and Tumorigenic Potential of Glioblastoma Stem Cells by Activating STAT3. *Cancer Cell.* 2011; 19(4):498–511. [PubMed: 21481791]
24. Hart JR, Liao L, Yates JR, Vogt PK. Essential role of Stat3 in PI3K-induced oncogenic transformation. *Proc. Natl. Acad. Sci. U. S. A.* 2011; 108(32):13247–13252. [PubMed: 21788516]
25. Adams JA. Activation Loop Phosphorylation and Catalysis in Protein Kinases: Is There Functional Evidence for the Autoinhibitor Model?†. *Biochemistry.* 2003; 42(3):601–607. [PubMed: 12534271]
26. Huse M, Kuriyan J. The Conformational Plasticity of Protein Kinases. *Cell.* 2002; 109(3):275–282. [PubMed: 12015977]
27. Bose R, Molina H, Patterson AS, Bitok JK, Periaswamy B, Bader JS, Pandey A, Cole PA. Phosphoproteomic analysis of Her2/neu signaling and inhibition. *Proc. Natl. Acad. Sci. U. S. A.* 2006; 103(26):9773–9778. [PubMed: 16785428]
28. Biscardi JS, Maa M-C, Tice DA, Cox ME, Leu T-H, Parsons SJ. c-Src-mediated Phosphorylation of the Epidermal Growth Factor Receptor on Tyr845 and Tyr1101 Is Associated with Modulation of Receptor Function. *J. Biol. Chem.* 1999; 274(12):8335–8343. [PubMed: 10075741]
29. Tice DA, Biscardi JS, Nickles AL, Parsons SJ. Mechanism of biological synergy between cellular Src and epidermal growth factor receptor. *Proc. Natl. Acad. Sci. U. S. A.* 1999; 96(4):1415–1420. [PubMed: 9990038]
30. Jacob KK, Whittaker J, Stanley FM. Insulin receptor tyrosine kinase activity and phosphorylation of tyrosines 1162 and 1163 are required for insulin-increased prolactin gene expression. *Mol. Cell. Endocrinol.* 2002; 186(1):7–16. [PubMed: 11850117]

31. Hikri E, Shpungin S, Nir U. Hsp90 and a tyrosine embedded in the Hsp90 recognition loop are required for the Fer tyrosine kinase activity. *Cell. Signalling*. 2009; 21(4):588–596. [PubMed: 19159681]
32. Yokoyama N, Miller WT, William E, Balch CJD, Hall A. Purification and Enzyme Activity of ACK1. *Methods Enzymol*. 2006; 406:250–260. [PubMed: 16472662]
33. Yokoyama N, Miller WT. Biochemical Properties of the Cdc42-associated Tyrosine Kinase ACK1 SUBSTRATE SPECIFICITY, AUTOPHOSPHORYLATION, AND INTERACTION WITH Hck. *J. Biol. Chem*. 2003; 278(48):47713–47723. [PubMed: 14506255]
34. Sorkin A, Helin K, Waters CM, Carpenter G, Beguinot L. Multiple autophosphorylation sites of the epidermal growth factor receptor are essential for receptor kinase activity and internalization. Contrasting significance of tyrosine 992 in the native and truncated receptors. *J. Biol. Chem*. 1992; 267(12):8672–8678. [PubMed: 1314835]
35. Sorkin A, Waters C, Overholser KA, Carpenter G. Multiple autophosphorylation site mutations of the epidermal growth factor receptor. Analysis of kinase activity and endocytosis. *J. Biol. Chem*. 1991; 266(13):8355–8362. [PubMed: 2022651]
36. Jorissen RN, Walker F, Pouliot N, Garrett TPJ, Ward CW, Burgess AW. Epidermal growth factor receptor: mechanisms of activation and signalling. *Exp. Cell Res*. 2003; 284(1):31–53. [PubMed: 12648464]
37. Wybenga-Groot LE, Baskin B, Ong SH, Tong J, Pawson T, Sicheri F. Structural Basis for Autoinhibition of the EphB2 Receptor Tyrosine Kinase by the Unphosphorylated Juxtamembrane Region. *Cell*. 2001; 106(6):745–757. [PubMed: 11572780]
38. Holland SJ, Gale NW, Gish GD, Roth RA, Songyang Z, Cantley LC, Henkemeyer M, Yancopoulos GD, Pawson T. Juxtamembrane tyrosine residues couple the Eph family receptor EphB2/Nuk to specific SH2 domain proteins in neuronal cells. *EMBO J*. 1997; 16(13):3877–3888. [PubMed: 9233798]
39. Deckert M, Elly C, Altman A, Liu Y-C. Coordinated Regulation of the Tyrosine Phosphorylation of Cbl by Fyn and Syk Tyrosine Kinases. *J. Biol. Chem*. 1998; 273(15):8867–8874. [PubMed: 9535867]

Figure 1.

Phosphoproteomic analysis of MCF10A cells with *PIK3CA* mutations. (A) Diagram of the p110 α subunit encoded by the *PIK3CA* gene with the frequency of mutations found in large scale human sequencing studies. p85: p85 binding domain; RBD: Ras binding domain.

Modified from www.cbioportal.org. (B) Schematic depicting the strategy used for quantitative phosphoproteomic profiling of *PIK3CA* Ex9 and Ex20 knock-in mutant cells.

Figure 2. Phosphotyrosine profiling results of MCF10A with *PIK3CA* mutations. (A) Density scatter plot of log₂-transformed phosphopeptide ratios (Ex9-KI/MCF10A vs Ex20-KI/MCF10A). Pearson correlation coefficient (R) is indicated. (B) Distribution of log₂-transformed phosphopeptide ratios (Ex9-KI/MCF10A and Ex20-KI/MCF10A). (C) Number of regulated proteins found in enriched signaling pathways (Modified Fisher's exact P value < 0.05). (D) Distribution of log₂-transformed ratios (Ex9-KI/MCF10A and Ex20-KI/MCF10A) of all proteins quantified in the total proteome analysis.

Figure 3.

Widespread modulation of the kinome by *PIK3CA* mutations. Phylogenetic tree of protein kinases is denoted with kinases identified in phosphoproteomic profiling. A color-coded site regulation pattern is shown in the form of a circle divided into two parts. The top half represents the fold change of phosphorylation sites identified in Ex-9-KI cells, and the bottom half represents Ex20-KI cells compared with MCF10A.

Figure 4.

Hyperphosphorylation of tyrosine kinases in the *PIK3CA* mutant cells. Representative MS spectra of tyrosine phosphopeptides belonging to EPHB4, EGFR, ABL1, and EPHA2 tyrosine kinases showing higher levels in the Ex9-KI and Ex20-KI cells compared with the parental MCF10A cells.

Figure 5.

Site-specific regulation of tyrosine kinases by *PIK3CA* mutations. Diagram representations of receptor tyrosine kinases (A) and nonreceptor tyrosine kinase (B) denoted with phosphorylation sites found to be regulated in either Ex9-KI (top half of circle) or Ex20-KI (bottom half of circle).

Figure 6.

Validation of phosphoproteomic results. Western blot analysis using phospho-specific antibodies against EGFR, EPHA2, MET, EFNB1, and ERBB2 and the corresponding total protein antibodies. β -actin serves as a loading control.